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EVALUATION OF THE IMMUNOGENICITY, LATENCY AND SAFETY OF A NEW GENETICALLY ENGINEERED BOVINE HERPES VIRUS VACCINE

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"Evaluation of the Immunogenicity, Latency, and Safety of a New Genetically Engineered Bovine Herpes Virus Vaccine Candidate"

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Abstract/Summary

Bovine herpes type 1 (BHV 1) is an important pathogen in cattle of all ages. The best protection against viral disease is believed to be vaccination with a modified live virus vaccine, however most live BHV-1 vaccine viruses are not safe in very young animals or in pregnant females. We have genetically engineered a mutant BHV-1 virus which has diminished ability to spread cell-to-cell in tissue culture and has been shown to be safe when administered either intravenously or intrasnasally in newborn calves. We have also shown that the mutant virus produces antibody and other immune responses similar in magnitude to conventional BHV-1 vaccines, and that vaccination of young calves (6-8 weeks of age) is completely protective against a challenge with a virulent field isolate of BHV-1. Since most live BHV-1 vaccines establish latent infections in vaccinated animals, we examined immunosuppressed vaccinated calves for evidence for mutant virus recrudescence; no evidence for the persistence of the mutant virus was found. The final phase of this project has demonstrated the safety of the mutant virus when used as a vaccine in pregnant heifers.

Executive Summary

Bovine herpes type 1 (BHV-1) is an important cause of economic losses in cattle. There are a variety of disease syndromes associated with infection with BHV-1 including respiratory disease in cattle of all ages (infectious bovine rhinotracheitis), abortion in pregnant animals, and fatal multi-systemic disease in young calves. There are several commercially available vaccines incorporating BHV-1, some of which are comprised of killed virus or recombinant viral proteins, and others in which the virus is live but attenuated to be less virulent. The live virus vaccines are generally considered to be superior to killed preparations since replication of the virus in the host may provoke more comprehensive and effective protection than exposure to the antigens of killed virus or recombinant viral proteins. However, live virus BHV-1 vaccines have been found to be unsafe in pregnant cows (causing abortion) and in young animals (causing fatal multi-systemic disease). There is a need for live BHV-1 vaccines which are safe in young and pregnant animals.

Recently, as part of a study to elucidate the roles of various surface glycoproteins of BHV-1, we genetically engineered a BHV-1 virus with a mutation of the gB surface glycoprotein. The gB protein is thought to be important in the cell-to-cell spread of the virus within the infected host. The mutated virus has a small deletion in the gene for the gB protein which results in failure of the normal cleavage of the protein into multiple parts. The deletion mutant virus (herein termed the 4S gB deletion mutant) infects and replicates in bovine cell cultures similarly to the parent virus however has diminished ability to spread from an infected cell to other cells nearby.

The diminished ability of the 4S gB deletion mutant virus to spread cell-to-cell suggests that, after vaccination, local infection might occur which would act to provoke an effective immune response, but that the virus may not be able to spread cell-to-cell to cause disease or to cross the placenta into the fetus to cause abortion. Thus a 4S gB deletion mutant virus may offer the safety features of a killed virus with the better immunogenic properties of a live virus resulting in a superior vaccine which could be used in all animals.

In a previous study funded by SDAF the *in vivo* safety of the mutated BHV-1 was demonstrated in neonatal calves. Newborn calves in the first days of life have severely compromised immune functions thus would be expected to be highly susceptible to systemic infection if the virus retained virulent properties. Neonatal calves were fed colostrum free of BHV-1 antibodies, then, while less than 24 hours of age were administered BHV-1 by one of two routes, either intranasally (expected route of natural transmission) or intravenously (to maximize exposure of the virus to the tissues of the calf). The calves were given either the 4S gB mutant or the parent virus from which the deletion mutant was produced. The calves were euthanized at various time points following infection and tissues examined grossly and histologically for evidence of lesions and by immunohistochemistry and viral isolation techniques for the presence of BHV-1 in the tissues.

In all calves given the parent virus either by intravenous or intranasal inoculation there were histological lesions and evidence for viral replication and cell-to cell spread within the infected tissues. In contrast, in calves given the 4S gB deletion mutant virus there were no histological lesions in the tissues and no evidence (by immunohistochemistry) for virus in the tissues. In one calf, given the 4S gB mutant BHV-1 and killed after 4 days, virus was found in the lungs by viral isolation techniques. These studies demonstrated that the 4S deletion mutant gB BHV-1 virus has very limited ability to replicate in bovine tissues *in vivo* suggesting that the virus is a safe candidate for a modified live virus vaccine suitable for administration to even neonatal animals.

The objectives of the present studies were to further examine the potential of the 4S gB deletion mutant as a vaccine virus. In order to be an effective live virus vaccine, the mutant virus must replicate sufficiently within the infected host to provoke an immune response which is protective against challenge by virulent (naturally occurring) BHV-1 virus. Our data shows that all calves vaccinated with a single dose of the mutant BHV-1 develop antibody responses to the virus similar in magnitude to those produced in calves in response to vaccination with a conventional killed BHV-1 vaccine and about 1/2 the magnitude of the response seen in calves following a conventional live BHV-1 vaccination. The usual vaccination protocols for both live and killed virus vaccines specify 2 vaccinations since the initial immunization usually produces low level primary immunity. Following secondary immunizations, calves given the 4S gB deletion mutant produce antibody responses similar to the conventional commercial vaccines (both killed and modified live commercial vaccines used in this study produced similar secondary antibody responses).

In addition to the antibody response, live vaccine virus should be capable of eliciting a cell mediated immune response. Cell mediated immune responses may be critical in controlling disease associated with viral infection. The current belief concerning immunity to viral infections is that while antibodies may be important in providing protection from initial infection, following entry of the virus into the cells of the body, antibodies are relatively impotent in clearing virus from infected host cells. Once the virus has entered the host cells, the killing of these viral infected cells is largely regarded as the function of cells called cytotoxic T lymphocytes. Many researchers believe that only a live virus vaccine (or natural infection) can provoke the specific cellular immunity of the cytotoxic T lymphocyte. A goal of the present study was to determine, using a battery of immunological assessment techniques, whether vaccination with the BHV-1 mutant will provoke cell-mediated as well as antibody responses. The present studies showed that *in vivo* measures of cell mediated immunity as demonstrated by cell blastogenesis, cell cytotoxicity and gamma interferon release assays were detectable in calves vaccinated with the 4S gB deletion mutant. However the tests of cell mediated immunity employed did not clearly distinguish superior responses, as would be expected, in animals given live virus vaccines compared to those given killed virus preparations. In both

experiments there were higher gamma interferon release assay results in calves given the 4S and/or commercial modified live virus vaccine compared to calves given killed virus vaccine and/or unvaccinated control calves, however, blastogenesis responses and delayed type hypersensitivity responses were slightly higher in cattle given the killed vaccine virus. The cell cytotoxicity assays did not distinguish among the groups.

While the results of laboratory tests of immunity often correlate well with protection against disease, there are many instances in which animals develop disease in spite of laboratory evidence of "protection" and many other instances in which animals without laboratory evidence for immunity appear to be solidly immune to virus challenge. To assess the actual protection from disease, we also determined whether the immune responses provoked by mutant BHV-1 vaccination protected against challenge with a virulent isolate of BHV-1. Unvaccinated control calves developed fevers which persisted for up to 6 days, exhibited erosive and ulcerative nasal lesions and shed BHV-1 in their nasal secretions for up to 10 days following aerosol challenge with a field isolate of BHV-1. In contrast, all animals vaccinated with the 4S gB deletion mutant were completely protected from the disease. In the group of animals vaccinated with the conventional commercial killed BHV-1 vaccine one animal developed nasal mucosal ulceration however there was no other clinical or laboratory evidence for disease. Thus the challenge study demonstrates solid protection after vaccination with the 4S gB mutant but not with the commercial killed vaccine virus.

BHV-1 is a herpes virus and like other herpes viruses has the propensity to become latent within the cells of the host following control of the initial infection. It is desirable that live virus BHV-1 vaccine candidates be completely eliminated from the host following a brief infection after vaccination. However, current live virus BHV-1 vaccines may undergo spread to the neurological tissues of the vaccinated animal and may establish persistent latent infections in these tissues. In times of immunosuppression the latent vaccine virus may be reactivated to produce infectious virus which may cause disease in either the host or in in-contact animals (especially of concern is the risk of abortion in in-contact pregnant females). Since the 4S gB BHV-1 mutant is crippled in its ability to spread cell-to cell it is expected that the virus will not spread from the initial injection sites to establish latent infections in neurological tissue. One of the goals of this study is to determine, using steroid-induced immunosuppression, whether the 4S gB mutant BHV-1 can cause latent infections. Calves, previously vaccinated with the 4S mutant, were tested following steroid-induced immunosuppression for evidence of recrudescence of the 4S gB deletion mutant. Immunoprecipitation studies on the viruses isolated from the calves showed evidence in 4/4 control (unvaccinated, challenged with native BHV-1) calves, 2/4 calves vaccinated with commercial killed BHV-1 vaccine and challenged with native BHV-1 and 2/3 calves vaccinated with the 4S gB deletion mutant BHV-1 vaccine for recrudescence of native BHV-1, but there was no evidence for expression of the mutated protein suggesting that the 4S gB mutant virus did not establish latent infections.

The final study examined the safety of the mutant virus in pregnant heifers. The most convenient protocol for administration of vaccines to beef cattle is in the fall at the time of weaning. At this time, the cows and heifers in the herd are usually pregnant, thus there is the need to ensure that the vaccine virus does not spread across the placenta to injure the fetus (resulting in fetal death and abortion). The final phase of these studies examined the safety of the mutant virus (administered as a vaccine) in pregnant females. It was our hypothesis, based on our previous findings of the extremely limited demonstration of viral replication in highly vulnerable neonates it is anticipated that the 4S deletion mutant would not spread to the bovine fetus and thus will be safe in pregnant females. In 10/10 pregnant females given up to 40 times the normal vaccine dose, pregnancies were maintained for over 5 months (5/10 animals have delivered healthy calves and the others are heavily pregnant).

Technical Report

The first phase of the study examined the immune responses to immunization with the deletion mutant BHV-1 virus. The immune responses following vaccination were studied in two experiments. In the first experiment, twelve newborn dairy calves were removed from their dams at birth and isolated in the animal housing facilities at the Western College of Veterinary Medicine. The calves were fed 2 litres of colostrum negative for antibodies to BHV-1 (tested by ELISA) to ensure that calves would have normal maternal protection to most pathogens but without interference of maternal BHV-1 antibodies to specific vaccination. Calves were blood tested for BHV-1 antibodies 48 hours following the second colostrum feeding to ensure no suckling had occurred prior to isolation and that the colostrum fed was indeed BHV-1 antibody negative.

Once all the calves had reached ages greater than one month they were stratified on the basis of age into two groups (calves greater than 2 months of age, n=6 and calves less than 2 months of age, n=6) then randomized into one of 3 groups. Since the calves ranged in age from just over 1 month to more than 2 months this stratification was designed to control for any effect of age on the immune response. Calves in group one (n=4) received conventional, commercially-available killed BHV-1 vaccine (Triangle II, Ayerst Laboratories Ltd.), calves in group 2 (n=4) received a conventional live virus BHV-1 vaccine (BoviShield, Pfizer Animal Health Ltd.) and calves in group 3 (n=4) received the gB deletion mutant BHV-1.

The 4S gB deletion mutant BHV-1 virus was grown in cell culture in a bovine kidney epithelial cell line in Dulbeccos Modified Eagles Media supplemented with 5.0 % equine or fetal bovine sera. When infected cells demonstrated maximal cytopathic effects the flasks were frozen at -70 deg C then thawed at 37 deg C. The cell debris was pelleted by centrifugation, then the supernatant transferred to a sterile bottle and the cell pellet and debris resuspended in 5.0 mLs sterile water. The resuspended material was disrupted by sonication, pelleted by centrifugation and the supernatants mixed and aliquoted into 5.0 mLs volumes. Aliquots of the viruses were stored at -70 deg C, then thawed immediately prior to use.

The amount of infectious virus was verified in the thawed aliquots of the 4S gB BHV-1 mutant virus by plaque formation assays. The 4S gB mutant virus grew to titres of 6.6×10^5 pfu/mL. Similar plaque formation assays performed with the commercial live virus vaccine demonstrated 3×10^4 pfu/mL. Calves receiving the 4S gB deletion mutant virus were given 2 mLs of virus (total dose of 1.3×10^6 pfu) by intramuscular injection. The commercial vaccine viruses were administered according to the manufacturers instructions also by intramuscular injection.

Prior to the first immunization, blood was collected from each calf and serum was harvested for day 0 testing for antibodies to BHV-1 and for baseline data for cell mediated immune responses. Calves were tested for blastogenesis responses to BHV-1, for specific cellular cytotoxicity to BHV-1 infected cells using a chromium release assay and a gamma interferon release assay. There was no evidence for pre-existing antibodies to BHV-1 or for specific cell-mediated immune responsiveness to BHV-1 antigens prior to the immunizations.

Twenty-one days after the initial vaccination and seven and twenty-one days after the second vaccination, the calves were blood sampled and serum collected for antibody titres. ELISA and serum neutralization antibody testing of these samples showed the results shown in appendix 1. The data demonstrates that ELISA antibody titres in response to a single immunization were remarkably uniform within each of the 3 groups. The calves receiving the conventional modified live virus vaccine had the highest antibody responses while those receiving the conventional killed and 4S gB deletion mutant virus had similar (and lower magnitude) responses. After initial immunization, calves given the commercial modified live virus vaccination had moderate antibody titres while those receiving the 4S gB deletion mutant or the commercial killed virus vaccine had low titres. However, following the second vaccination ELISA antibody titres measured at 7 and 21 days post-vaccination were moderate in magnitude in all calves in all of the groups (data also shown in appendix 1).

The sera collected at these time points were also tested for serum neutralizing (SN) antibodies, SN antibodies may reflect a subpopulation of the antibodies detected by ELISA and may be the critical component of the antibody response in preventing infection. There was no evidence for SN antibody prior to immunization; after the initial immunization there were low titres in calves given the killed vaccine and in those given the 4S gB deletion mutant while calves given the commercial live virus vaccine had moderate titres. Following the second vaccination, calves given the 4S deletion mutant developed and maintained (to day 21) moderate titres as did those given the two commercial vaccines.

The calves in this experiment were also tested for evidence of cell-mediated immunity to BHV-1 prior to and following primary and secondary immunizations. Calves were tested for blastogenesis responses to BHV-1, for specific cellular cytotoxicity to BHV-1 infected cells using a chromium release assay and a gamma interferon release assay. In addition, 7 days following the second immunization, calves were tested for evidence of delayed type hypersensitivity by intradermal injection of 100 µL of uninfected tissue culture supernatant or tissue culture supernatant containing BHV-1 (5×10^5 pfu). The data for the results of the tests of cell-mediated immunity are also summarized in Appendix 1.

Blastogenesis responses to BHV-1 antigen were low in all groups, a positive response was defined as a stimulation index equal to or greater than 2. There were no responders in calves vaccinated with the 4S gB deletion mutant, there was one responder in calves vaccinated with the commercial modified live virus vaccine, and 2 responders in the group vaccinated with the killed virus vaccine. The results of BHV-1 antigen-specific gamma-interferon release assays (7 days following the second vaccination) demonstrated only very low reactivity in all calves given the conventional killed BHV-1 vaccine, while 2/4 calves given the 4S deletion mutant and 3/4 calves given the conventional live BHV-1 vaccine had moderate or high responses. The cytotoxicity against BHV-1 infected target cells, measured with a chromium release assay, demonstrated variable levels of killing of BHV-1 infected cells in all calves vaccinated with the 4S gB deletion mutant or with the modified live commercial vaccine and in 2/4 calves vaccinated with the commercial killed virus vaccine following secondary immunization. The delayed type hypersensitivity skin test also showed variable responses, low and moderate responses were found in animals given the 4S gB mutant and the commercial live virus vaccine. The highest responses were in the group given the killed vaccine, two animal had a moderate responses, two animals had high responses.

In summary, the first experiment clearly demonstrated the ability of the 4S gB deletion mutant vaccine candidate virus to provoke antibody responses similar to the commercially available live and killed virus BHV-1 vaccines. The results of the testing for cell mediated immunity, while variable, also demonstrated that vaccination with the 4S mutant provoked cell mediated responses. Surprisingly, there were no obviously superior results in animals vaccinated with the live virus (either the 4s gB deletion mutant or commercial vaccine virus) compared to those found in animals vaccinated with the killed virus vaccine.

The design of the second experiment of virus immunogenicity included testing another group of calves for *in vitro* evidence of immune responses following vaccination and most importantly, for evidence of protection following challenge with virulent BHV-1. Twelve calves were conditioned similarly to those described in the first experiment (calves were attained within hours of birth and fed BHV-1-negative colostrum).

At approximately 7-8 weeks of age the calves were divided into 3 groups (n = 4/grp) and received 2 intramuscular vaccinations at 3 week intervals with either 1.3×10^6 pfu of the 4S gB deletion virus in 2 mL volume or with a conventional killed BHV-1 vaccine (Triangle III, Ayerst Laboratories Ltd.) used according to the manufacturers directions. The third group of calves were unvaccinated. All calves were tested for antibody production and for evidence of cell -mediated immunity, using the test methods previously described, prior to initial immunization, 3 weeks after the secondary immunization, and 4, 7 and 10 days after challenge with a virulent BHV-1.

Three weeks following the second vaccination, all calves were challenged with a virulent isolate of BHV-1 obtained from the Diagnostic Virology Laboratory of the Western College of Veterinary Medicine (isolate V95 1809 was isolated from a 6 month old calf dying from severe pneumonia associated with BHV-1). The challenge virus was propagated in tissue culture as previously described for the propagation of the 4S gB deletion mutant. For viral challenge, calves were administered, by face mask, 1×10^7 pfu of cell passage 2 virus in a final volume of 10 mLs of tissue culture media using a Devilbiss Ultra-Neb-99, ultrasonic nebulizer. The average time of each administration was approximately 20 minutes.

On the day of challenge and daily thereafter for 10 days calves were inspected and clinical assessment performed by two veterinarians (Drs Keith West and Deborah Haines) who were blinded to the identity of the groups. Calves were assessed for fever, depression, nasal discharge and a point light source used to examine the nasal cavity for evidence of hyperemia, vesicles, erosions or ulcers (the scoring system used to assess the calves was previously developed by Dr R Harland and has been used extensively in assessing BHV-1 challenges).

Nasal swabs were collected by deep insertion of a 9 inch cotton tipped sterile swab into the medial meatus of one nostril of each calf on days 0, 2, 3, 4, 5, 6, 7, 8, 9 and 10 following challenge. The swabs were placed immediately in 1 mL of transport media (DMEM containing 10 % FCS, 0.3 M $MgSO_4$, 0.4 M sucrose and antibiotics). Nasal swabs were stored frozen at -70 C until processed for viral isolation. Nasal virus shedding was quantified by determining the $TCID_{50}$ of the swab-containing transport media using a virus isolation technique with a sensitivity of 12.5 pfu/mL.

Blood was collected on days 0 (just prior to challenge), 4, 7 and 10 and tested for complete and differential cell counts. Blood was similarly collected at these time points and tested for evidence of cell immunity to BHV-1 using blastogenesis, gamma interferon release and chromium release cytotoxicity assays. In addition, serum samples were obtained on these days for assessment of antibody responses. Calves were tested for *in vivo* evidence of cell-mediated immunity to BHV-1 on day 0 by delayed type hypersensitivity skin testing. Each calf was tested by intradermal injection of 5×10^6 pfu partially-purified BHV-1 in 100 uL of tissue culture media in the right side cervical skin. The left side of the calf was similarly injected with a similar volume of uninfected cell culture tissue extract. The thickness of the skin was measured prior to injection of the BHV-1 or control antigen and 24, 48 and 72 hours later.

Antibody responses, measured by ELISA are shown in Appendix 2. There were no detectable antibodies in the unvaccinated control calves until 10 days following challenge with the virulent BHV-1 at which time all control calves had low antibody responses. In contrast, all vaccinated calves (both 4S gB deletion mutant and commercial killed virus vaccinees) had moderate antibody responses prior to

challenge and on day 4 following challenge. By day 7 following vaccination, there were anamnestic immune responses resulting in high antibody levels in all calves in both vaccinated groups.

Summaries of data pertaining to the measurements of cell mediated immunity are also shown in Appendix 2. The blastogenesis responses to BHV-1 were negative in the control (unvaccinated calves), there were low positive responses in 3/4 calves vaccinated with the 4S deletion mutant and highly variable (low to very high) responses in all calves vaccinated with the commercial killed virus vaccine.

The BHV-1 antigen driven gamma interferon release prior to challenge (tested following the secondary immunization) was highly variable. Three of 4 unvaccinated calves and all calves in the 4S deletion mutant vaccinated groups had detectable responses while all calves in the group vaccinated with the killed virus vaccine had low or undetectable responses. Ten days following challenge with virulent BHV-1 all calves in the group vaccinated with the 4S virus had moderate to high responses while those in the other groups (with the exception of one unvaccinated animal) had low or undetectable responses.

The levels of cellular cytotoxicity to chromium labeled BHV-1 infected targets, measured 14 days following the secondary immunization, was lower than in the previous experiment. One control calf had a positive response (defined as killing in excess of 10% of cells), 3/4 calves given the commercial killed virus vaccine and 2/4 calves given the 4S gB deletion mutant vaccine had positive responses.

Delayed type hypersensitivity skin responses were, as in the first experiment, greatest in calves vaccinated with the killed virus vaccine followed by those vaccinated with the 4S mutant. There were also responses, howbeit lower, in the control unvaccinated calves. The latter result may be a reflection of the higher antigen dose used for intradermal challenge compared to that employed in the first experiment.

The challenge with the virulent isolate of BHV-1 produced clinical evidence of infectious bovine rhinotracheitis (IBR) in all 4 of the unvaccinated calves. The results of the post challenge assessments are shown in Appendix 3. On day 1 post challenge, the average body temperature in the control calves was in excess of 104.5 degrees Fahrenheit, the temperatures in these calves remained elevated above normal (greater than 102.5 degree F) until day 7 post challenge. In contrast, there was no evidence of fevers in any of the calves given either the 4S gB deletion mutant BHV-1 vaccination or the commercial killed virus vaccine. In addition, all calves in the control, unvaccinated group developed (by day 3) variable sized erosions and ulcers of the nasal mucosa. There was no nasal lesions observed in any calves vaccinated with the 4S gB deletion mutant. In one calf vaccinated with the killed commercial vaccine a large (> 2 mm) nasal ulcer was observed on day 3-5 post challenge. BHV-1 was recovered from nasal swabs taken from all control calves on days 2, 3 and 5 and from a variable number of the calves in this group at

other time points. In one control calf BHV-1 virus was recovered, still in large amounts on day 10 post infection. There was no evidence of viral shedding in any of the vaccinated calves.

Complete and differential blood counts on days 1, 4, 7 and 10 demonstrated a mild leukocytosis due to a neutrophilia in all groups of calves.

The ultimate test for vaccine performance is protection from challenge with virulent virus. The 4S gB deletion mutant virus provided complete protection from clinical symptoms and shedding of infectious virus following challenge with a virulent isolate of BHV-1. The killed commercial vaccine used in this study also provided protection from most symptoms however the observation of nasal mucosal ulceration in one calf suggests that the protection may not have been complete.

These studies demonstrated that measurable immune responses are provoked following immunization with the 4S gB deletion mutant vaccine candidate. The primary antibody responses were very uniform within the vaccinated calves suggesting that the levels of replication did not vary greatly from calf to calf. The magnitudes of the primary antibody responses were lower than those seen with the conventional live virus BHV-1 vaccine and similar to those seen with the conventional killed BHV-1 vaccine. This is compatible with the expectation that the amount of replication of the mutant virus is more limited than that of the conventional live virus vaccine. The responses to secondary immunization (and to challenge with virulent virus) showed moderate titre antibody responses in all groups of vaccinated calves demonstrating that priming for anamnestic antibody responses occurred equally effectively with the 4S gB deletion mutant and with either the commercial live virus or killed virus vaccine.

It is the conventional wisdom and there is data in other studies that suggest that live virus vaccines will provide superior cellular immunity to killed virus vaccines. In this study the laboratory testing for cell-mediated immune responses were highly variable and while providing evidence of cellular responses in 4S deletion mutant vaccinated calves, in most assays there was no clear evidence for superior responses in animals vaccinated with the live virus vaccines (either the 4S deletion mutant or the commercial live vaccine) compared to those responses seen in animals vaccinated with the commercial killed virus vaccine. The failure to show enhanced cellular immunity with live virus preparations compared to killed in the present studies may relate to the relatively poor ability of the available tests for cellular immunity to reflect the immune status within the animal. The inability to distinguish live and killed vaccine virus effectiveness may also be due to the relatively short time period between vaccination and testing/virus challenge. The time periods in these experiments were necessarily short (3 weeks) due to the high costs of housing experimental calves, however, in the cattle industry the duration of protection is expected to be at least one year. It may be that since the killed vaccine

has not replicated in the host tissues the duration of immunity will be much briefer than with the live virus vaccines.

A property of herpes viruses is their ability to replicate in neuronal tissues and to develop latent infections in these tissues. Latent infections are important since latently infected animals, given clinical stress, may begin to replicate the virus and develop both clinical symptoms and more importantly, may shed the virus to in-contact animals in which it may not be safe (for example to pregnant females in which it may be abortigenic). An experiment was performed to examine the potential for latent infection of the mutant BHV-1 virus. In this study we examined steroid-induced recrudescence of BHV-1 in calves vaccinated with either the 4S gB deletion mutant virus, with a commercial killed BHV-1 vaccine or unvaccinated control calves; then challenged with a virulent BHV-1 virus. The calves used in this experiment were those used to test the immunogenicity and vaccine effectiveness; the vaccine and challenge protocols and results were described in the last report. For the purposes of the present experiment, it is important to recall that the challenge with the virulent isolate of BHV-1 produced clinical evidence of infectious bovine rhinotracheitis (fevers and nasal erosions and ulceration) in all 4 of the unvaccinated calves. In contrast, there was no evidence of fevers or other disease in any of the calves given either the 4S gB deletion mutant BHV-1 vaccination or the commercial killed virus vaccine. BHV-1 was recovered from nasal swabs taken from all control calves on days 2, 3 and 5 and from a variable number of the calves in this group at other time points. In one control calf BHV-1 virus was recovered, still in large amounts on day 10 post infection. There was no evidence of viral shedding in any of the vaccinated calves.

To assess the ability of the 4S gB deletion mutant to establish a latent infection 12 weeks following challenge calves were immunosuppressed by intramuscular administration of 0.1 mg/kg dexamethasone once daily for 5 days. There were 2 methods used to assess recrudescence of BHV-1. The first assay was serological measurement of antibodies to determine evidence of increase in antibody titre consistent with viral antigen expression (and therefore recrudescence). The calves were tested for serum antibody titres to BHV-1 by ELISA (as previously described) on days 0, 6, 11, 23. The second assay was isolation of virus from nasal swabs to determine shedding of recrudescent virus. For this assay, nasal swabs were collected by deep insertion of a 9 inch cotton tipped sterile swab into the medial meatus of one nostril of each calf on days 0, 5, 6,7,8,9,10,11,12. The swabs were placed immediately in 1 mL of transport media (DMEM containing 10 % FCS, 0.3 M $MgSO_4$, 0.4 M sucrose and antibiotics). Nasal swabs were stored frozen at -70 C until processed for viral isolation. Nasal virus shedding was quantified by determining the $TCID_{50}$ of the swab-containing transport media using a virus isolation technique on EBTR (embryonic bovine trachea) cells with a sensitivity of 12.5 pfu/mL.

The results of testing for seroconversion showed increases in antibody titres (implying recrudescence of BHV-1) in 4/4 control animals, in 3/4 animals vaccinated with the commercial killed BHV-1 preparation and in 1/3 animals vaccinated with the 4S gB deletion mutant. Unfortunately one of the 4S vaccinates was lost to the experiment due to unrelated reasons.

The testing of nasal swabs for virus isolation showed positive results in 4/4 control animals on multiple days and in 2/4 animals vaccinated with the killed BHV-1 vaccine (one animal on multiple days and one animal one day only) and in 2/3 animals vaccinated with the 4S gB deletion mutant (one animal on multiple days and one animal one day only).

Since all animals had been challenged with virulent BHV-1 to determine the nature of the recrudesced virus, immunoprecipitaton tests were performed with the viral isolates from 4S vaccinated calves to determine if the isolates were the 4S gB deletion mutant or native (unmutated) BHV-1. Briefly, virus isolated from the 2 4S vaccinated calves were inoculated in cell culture and the cells were radiolabelled. Following incubation, the cells were harvested and sonicated, a monoclonal antibody directed to the gB BHV-1 protein added, the virus/monoclonal antibody eluted and precipitated with immunobeads tagged with antibodies to murine immunoglobulin, then the viral proteins were separated by electrophoresis. The autoradiographs of the electrophoresis results demonstrated that both isolates from the 4S gB deletion vaccinated calves expressed the cleaved gB protein (with molecular weights approximately 190K, 90 K and 50K) rather than the 4S gB vaccine virus protein which is uncleaved and yields a single band at 190K). These results demonstrate that the 4S gB vaccinated calves were shedding the challenge native BHV-1 virus rather than the 4S gB mutated vaccine virus.

Five days of steroid therapy caused recrudescence of BHV-1 in all unvaccinated control calves 12 weeks following exposure to virulent BHV-1. This demonstrates that without vaccination it is to be expected that BHV-1 exposed calves will both develop acute clinical disease and latent persistent infections which in times of stress can recrudesce and shed to infect other animals. While in a previous experiment we demonstrated that 3/4 calves vaccinated with a killed BHV-1 vaccine had clinical protection from disease, in the present study, following immunosuppression, it was shown that 3/4 of these calves seroconverted to BHV-1 and 2/4 shed BHV-1 in nasal secretions indicting that in spite of few signs most calves vaccinated with the killed vaccine developed latent infections. Similarly while 4/4 4S vaccinated calves were protected from clinical symptoms after challenge, following immunosuppression, 2/3 mutant-vaccinated calves tested had evidence for seroconversion and viral shedding indicting the presence of latent infection with the challenge virus. These data suggest that protection from clinical disease provided by the 4S vaccine will not prevent the establishment of latent infections following virulent BHV-1 exposure.

The major goal of this experiment was to test immunosuppressed calves to determine if the 4S gB deletion mutant per se established latent infections in vaccinated animals. While 2/3 4S vaccinates shed BHV-1 following immunosuppression, this was shown by immunoprecipitation studies to be native BHV-1, therefore there was no evidence that the 4S gB deletion mutant established latent infections in vaccinated animals. While these studies show that the 4S mutant will not prevent the development of latent infections with field isolates it suggests that the vaccine itself may not become latent. While much larger groups of animals would need to be tested to verify this suggestion, the finding has relevance if the vaccine is to be used in countries in which there is a BHV-1 eradication program.

The final experiment of this study examined the safety of the 4S mutant virus when used as a vaccine in pregnant cattle. A life virus BHV-1 vaccine suitable for animals of all ages must be safe in pregnant females to be useful. For this study, blood was collected from groups of heifers belonging to beef cattle producers in southern Saskatchewan and tested for serological evidence of antibodies to BHV-1. Two herds with high numbers of seronegative heifers were identified, resampled and retested to confirm antibody negative status and 12 animals purchased and maintained on a pasture isolated from other cattle. In late spring the animals were heat synchronized by hormone administration and bred by artificial insemination. Heifers showing subsequent evidence of heat were rebred. Six heifers conceived in 2 breedings and were retained in the experiment, six heifers were open after 2 breedings and were sold and 4 additional heifers from the same farm, naturally bred and confirmed to be seronegative to BHV-1 were purchased.

The heifers were moved from pasture to corral facilities at the Western College of Veterinary Medicine early in the second trimester of pregnancy which is the time in which the fetus is considered most sensitive to BHV-1 induced abortion. All animals were confirmed pregnant and seronegative to BHV-1 on arrival at the WCVM. After a 2 week acclimatization period, the animals were again confirmed pregnant and seronegative. The heifers were then given intramuscular injections of the 4S mutant BHV-1 virus, 6 heifers were given 15 mLs of tissue culture fluid divided into 2 sites (right rump and left cervical area) and 4 heifers were given 7.5 mLs in the right rump. The vaccine virus preparation was 4S virus grown in tissue culture as previously described and tested by plaque formation assay. The inoculation was shown to contain 3.3×10^6 pfu/mL or in excess of 10^7 virons/heifer. Since the usual dose of a live virus vaccine is about 1×10^6 pfu this dose was about 20 and 40 times the usual dose. These high levels were given to maximize the test of the safety of the virus in these animals.

Following vaccination the heifers were monitored daily for evidence of clinical disease and/or fever. The genital area was inspected twice daily for evidence of discharge indicating abortion and the corral was carefully examined twice daily for evidence of fetal remains. The heifers were rectally examined once weekly for the next 14 weeks for confirmation of pregnancies. Blood samples were obtained 2

weeks after the vaccination and tested for evidence of antibodies to BHV-1 as previously described.

All animals seroconverted to BHV-1 following immunization, the titers however were moderate which implies that even the very high doses of 4S virus administered in this study will not be effective in producing high titer immune responses. There was no evidence of clinical symptoms associated with the vaccination. None of the heifers aborted, all have maintained their pregnancies for 16 weeks post immunization, five of the heifers have recently delivered full term healthy calves and 5 are heavily pregnant and soon due to calf at the time of this writing.

This series of studies has shown the safety of the 4S mutant BHV-1 virus in neonatal calves, in feeder calves and in pregnant heifers. The immune responses to the 4S virus have been demonstrated to be moderate and the efficacy in protecting calves against a respiratory challenge shown to be good. The data to date suggests that the 4S has potential to be used as live BHV-1 vaccine candidate.

Project Activities, Achievements, Papers and Presentations:

The results of this work and/or the previous work to develop the deletion mutant virus (funded by NSERC) and the test of it's safety in neonatal calves (funded by the SADF project #9400096) will not be published or presented until it is determined whether the virus has commercial vaccine value. Our method of assessment of the potential for commercial value will be the presentation of the results of the present studies to selected veterinary vaccine manufacturers for expressions of interest. If the vaccine proves to be of interest to industry and thus of commercial value it is likely that a patent will be sought, if the virus is not shown to be of commercial value then the results of these and the previous studies will be published in the veterinary scientific literature as the functional significance of the gB protein deletion mutation in the BHV-1 virus is of scientific value.

Indications for Further Technical or Commercial Development

The results to date suggest that the virus has the potential to be exploited commercially. There is currently only one live virus BHV-1 vaccine used in pregnant cattle (the temperature sensitive mutant BHV-1 used in Cattlemaster™ marketed by Pfizer Animal Health Inc.) The 4S gB deletion mutant is the first rationally designed BHV-1 virus which has a defined change which will enable it be both identifiable and safe.

Information of Benefit to Producers, Government or Industry

The studies completed in this report continue to suggest that the 4 S gB deletion BHV-1 mutant may have commercial potential as a live virus vaccine suitable for use in all ages of animals. This will be convenient as livestock owners will now be able to vaccinate all animals with a single live virus vaccine preparation and will be able to co-mingle vaccinated animals of all ages without the risk of inducing abortion.

If the 4S gB deletion mutant virus is utilized as a vaccine it is expected that royalty revenue will be payable to the University of Saskatchewan which will benefit the university and the province.

Personnel

Jaret Bogdan has been employed (Technician level III) for approximately 1/2 time for 2 months in the project to July 31/96. Carrie Konoby (Technician level II) has been employed full time from Oct/96-April/97 and part-time to August 97. Tim Slemp (2nd year veterinary student) was employed from May 98 to Sept 98 on this project.

Equipment

There has been no purchase of equipment (in excess of $500) for this project.

Project Developed Materials

None yet produced.

Project Photos

There are photographs of the BHV-1 challenge procedure in calves which can be made available upon request.

SADF Acknowledgment

The support of the SADF will be acknowledged when the results of these studies are released. If the results of the trials support the use of the mutated BHV-1 virus as a vaccine candidate the data will be held confidential for some time as the virus will probably be patented and any publicity of the results would jeopardize the awarding of a patent. If the results do not support the use of the mutated virus as a vaccine candidate the data will be published in the scientific literature as the biological properties of the mutated virus are of academic interest.

Expense Statement

The statement of expenditures has been forwarded directly to the SADF offices from the University of Saskatchewan Office of Research Services.

Appendices

Appendix 1: Results: Experiment #1

BHV-1 antibody levels measured by ELISA in calves vaccinated with the deletion mutant (4S), modified live virus (MLV) or killed (KV) vaccines.

BHV-1 serum neutralizing antibody scores in calves vaccinated with the deletion mutant (4S), modified live virus (MLV) or killed (KV) vaccines.

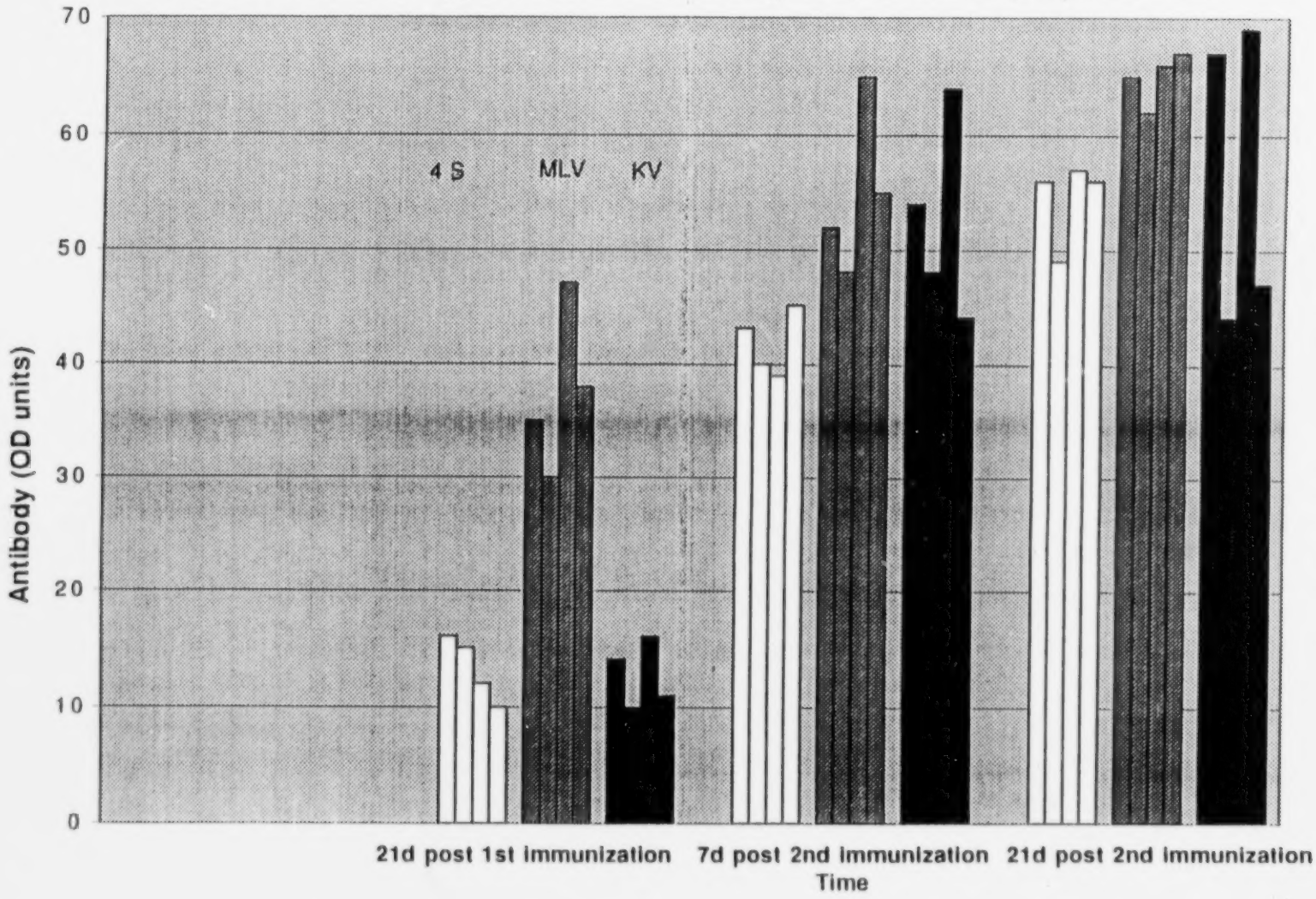
Blastogenesis responses to BHV-1 antigen in calves vaccinated with the deletion mutant (4S), modified live virus (MLV) or killed (KV) vaccines on day 7 following secondary immunization.

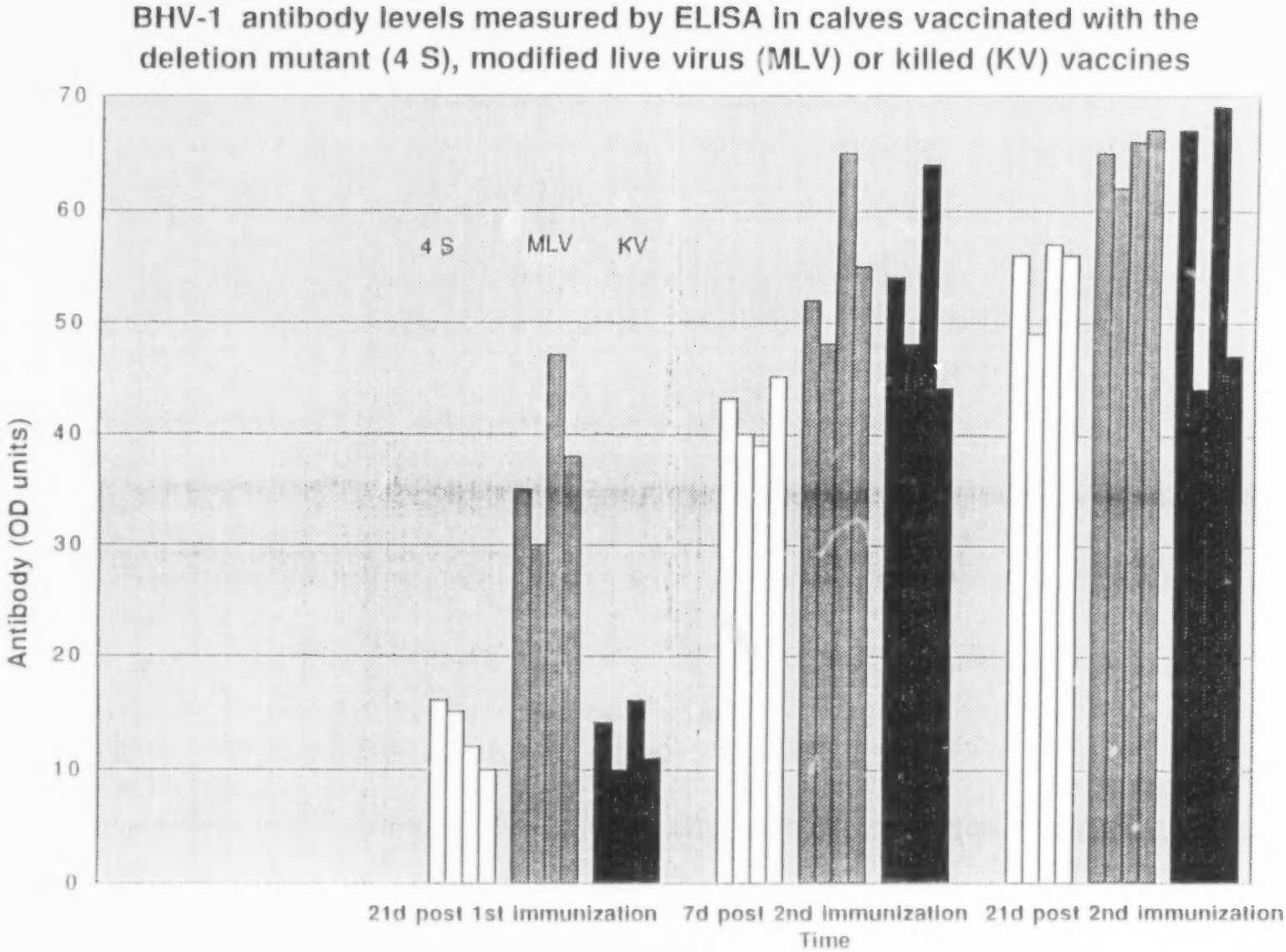
Interferon gamma responses to BHV-1 antigen in calves vaccinated with the deletion mutant (4S), modified live virus (MLV) or killed (KV) vaccines on day 7 following secondary immunization.

18 hour BHV-1 cytotoxicity assay (E:T 100:1), day 7 following secondary immunization in calves vaccinated with the deletion mutant (4S), modified live virus (MLV) or killed (KV) vaccines.

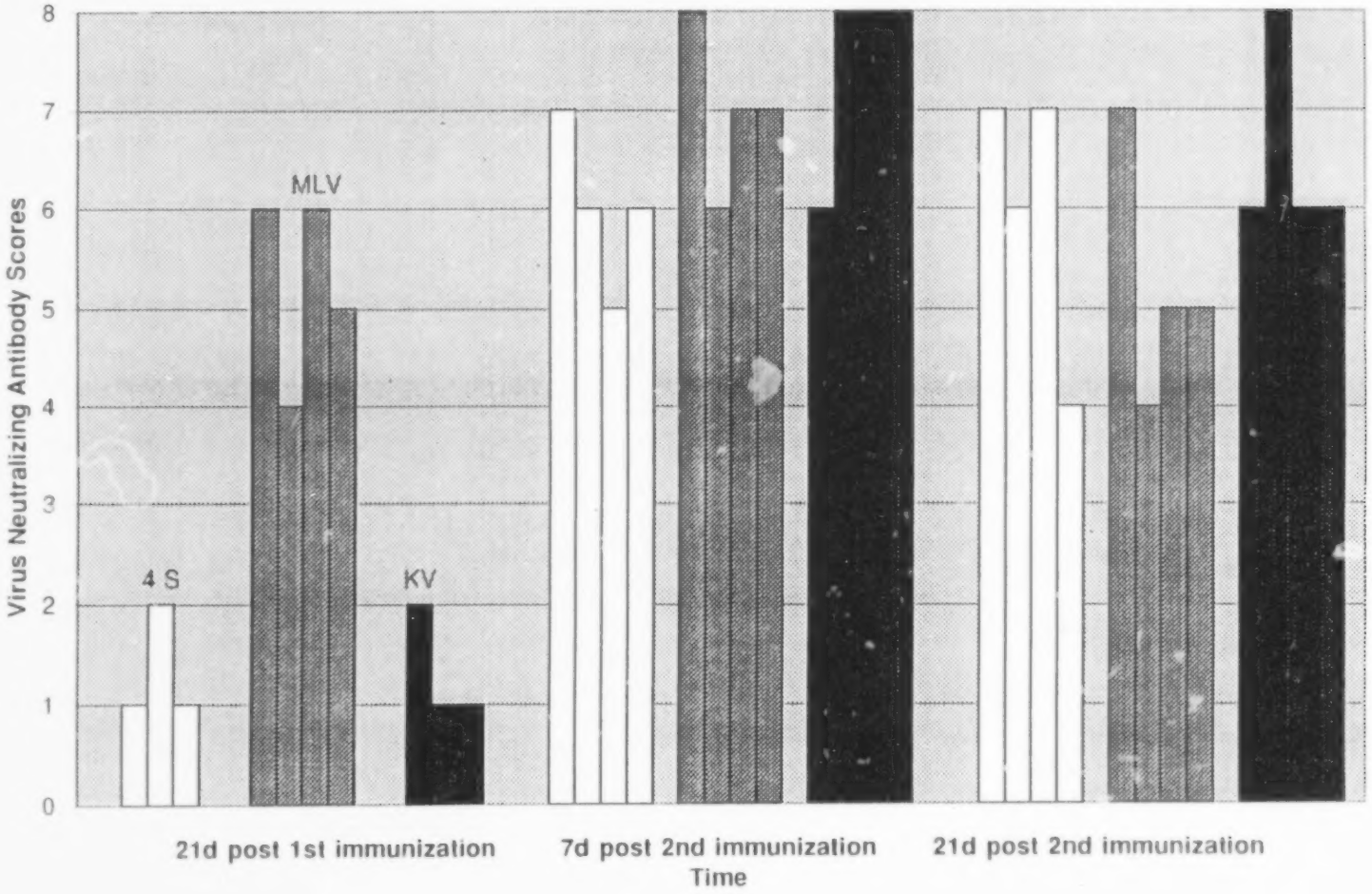
Delayed type hypersensitivity responses to BHV-1 antigen in calves vaccinated with BHV-1 deletion mutant (4S), modified live virus (MLV) or killed (KV) vaccines.

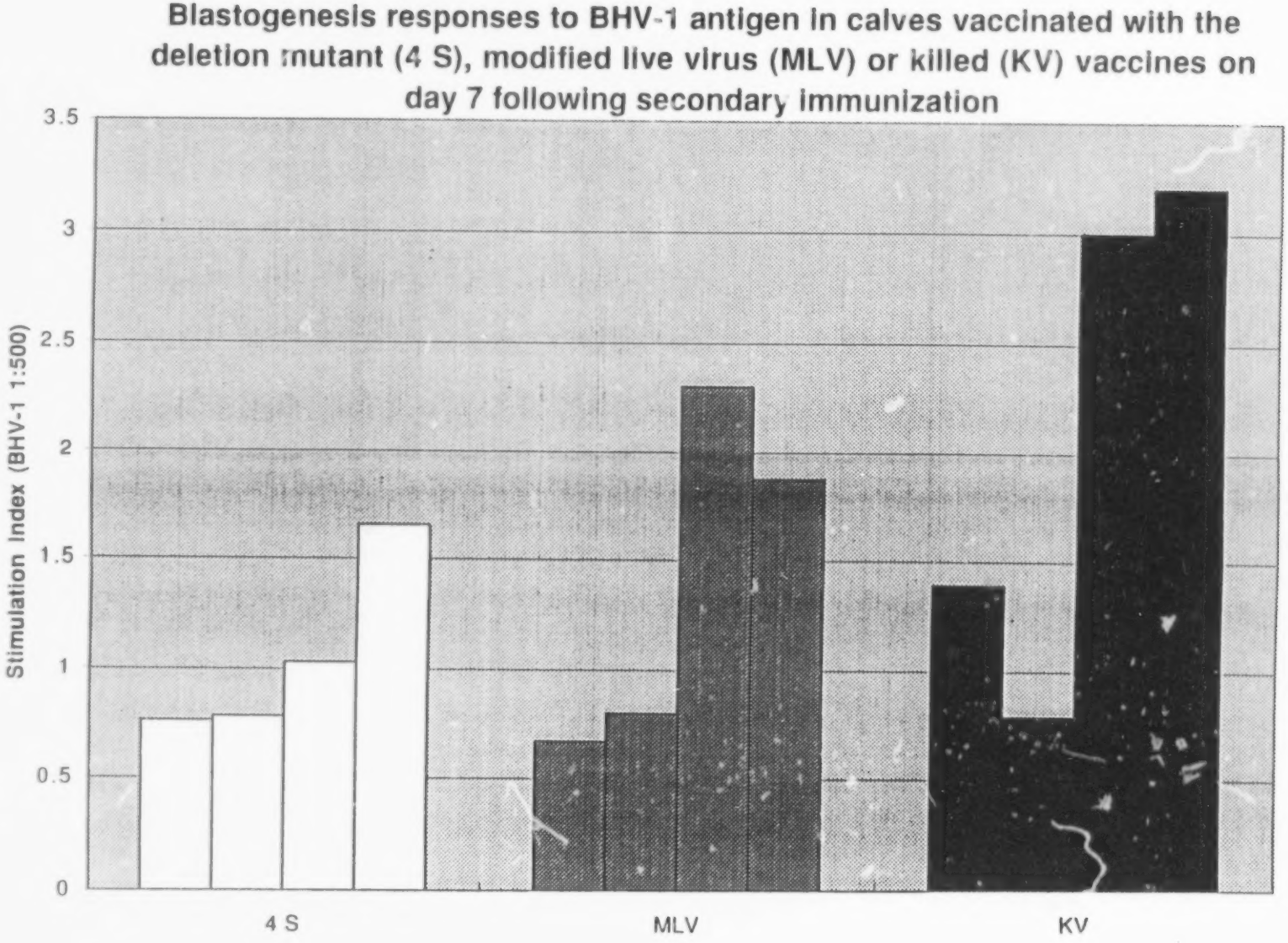
BHV-1 antibody levels measured by ELISA in calves vaccinated with the deletion mutant (4 S), modified live virus (MLV) or killed (KV) vaccines



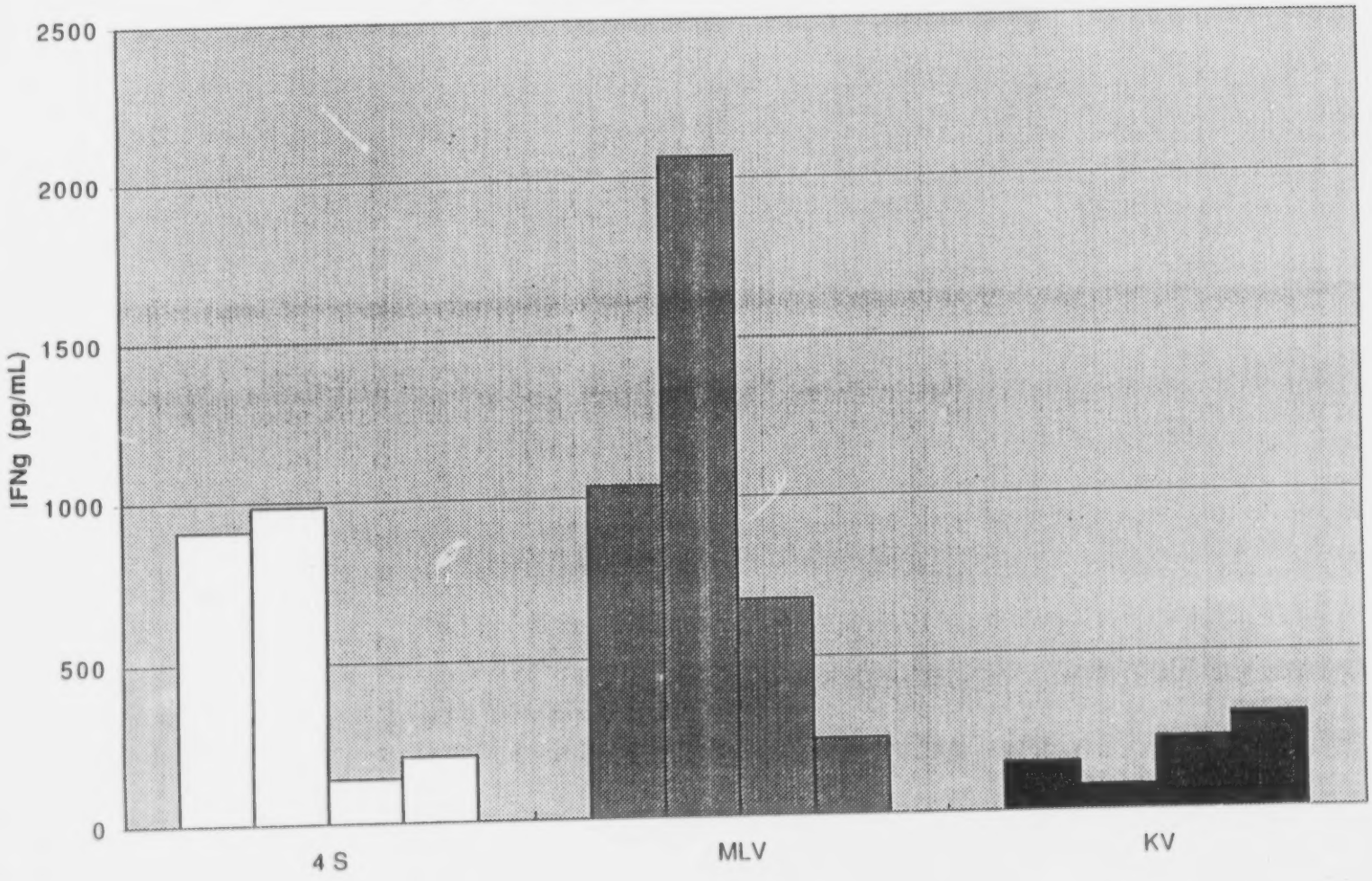

BHV-1 antibody levels measured by ELISA in calves vaccinated with the deletion mutant (4 S), modified live virus (MLV) or killed (KV) vaccines
Antibody (OD units)
70
60
50
40
30
20
10
0
4 S
MLV
KV
21d post 1st immunization
7d post 2nd immunization
21d post 2nd immunization
Time

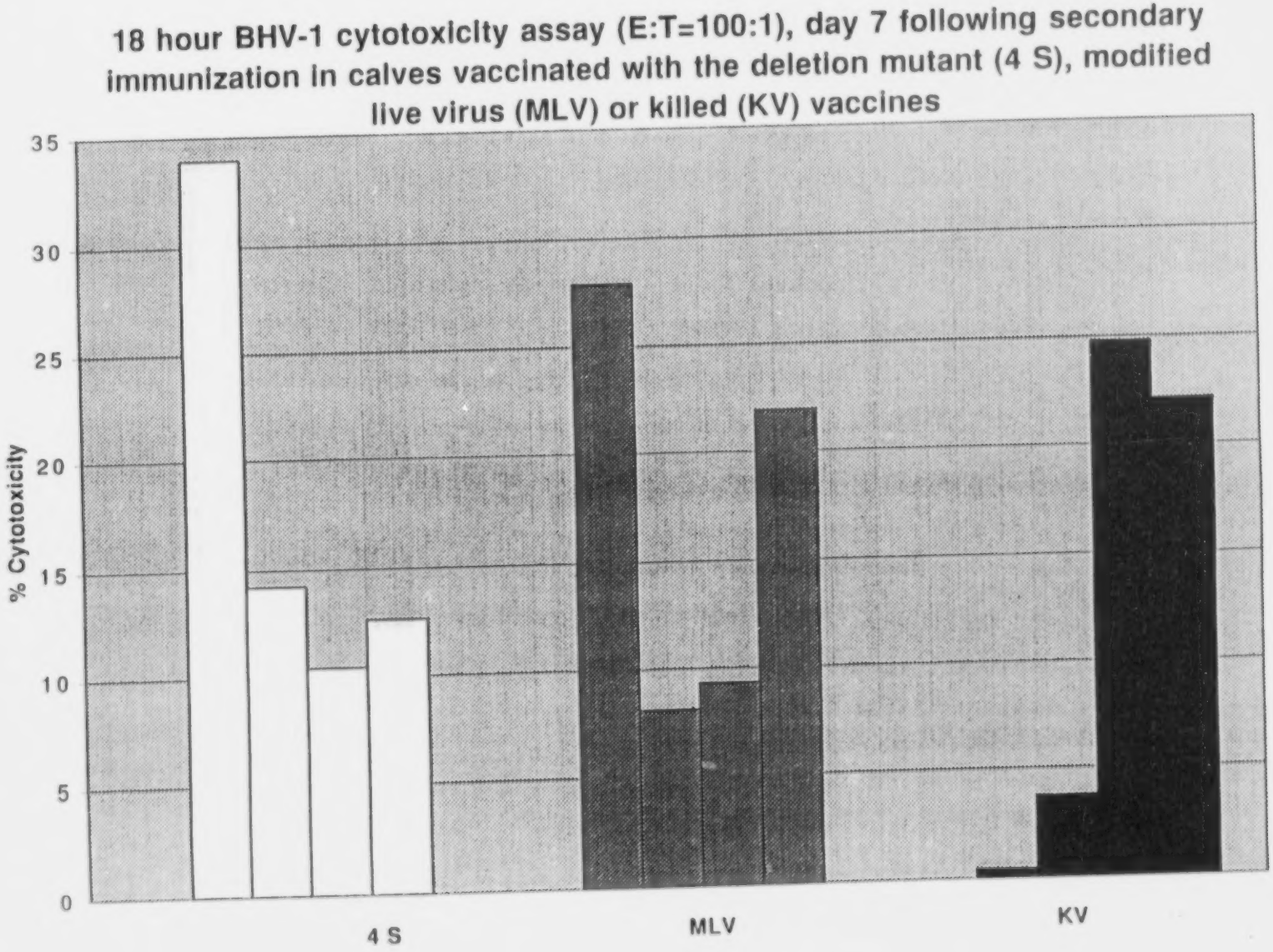
BHV-1 virus neutralizing antibody scores in calves vaccinated with the deletion mutant (4 S), modified live virus (MLV) or killed (KV) vaccines



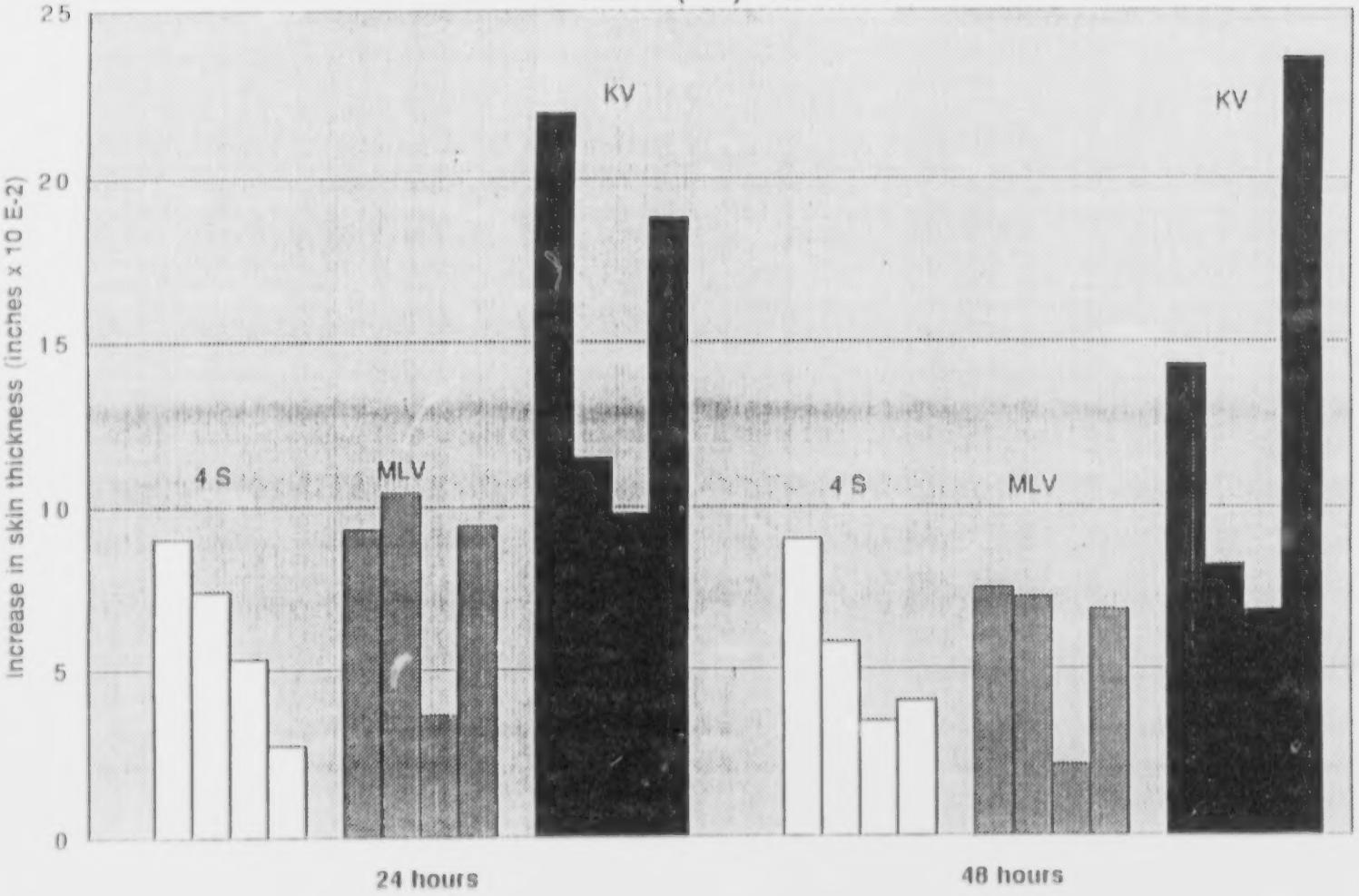

Blastogenesis responses to BHV-1 antigen in calves vaccinated with the deletion mutant (4 S), modified live virus (MLV) or killed (KV) vaccines on day 7 following secondary immunization
Stimulation Index (BHV-1 1:500)
3.5
3
2.5
2
1.5
1
0.5
0
4 S
MLV
KV

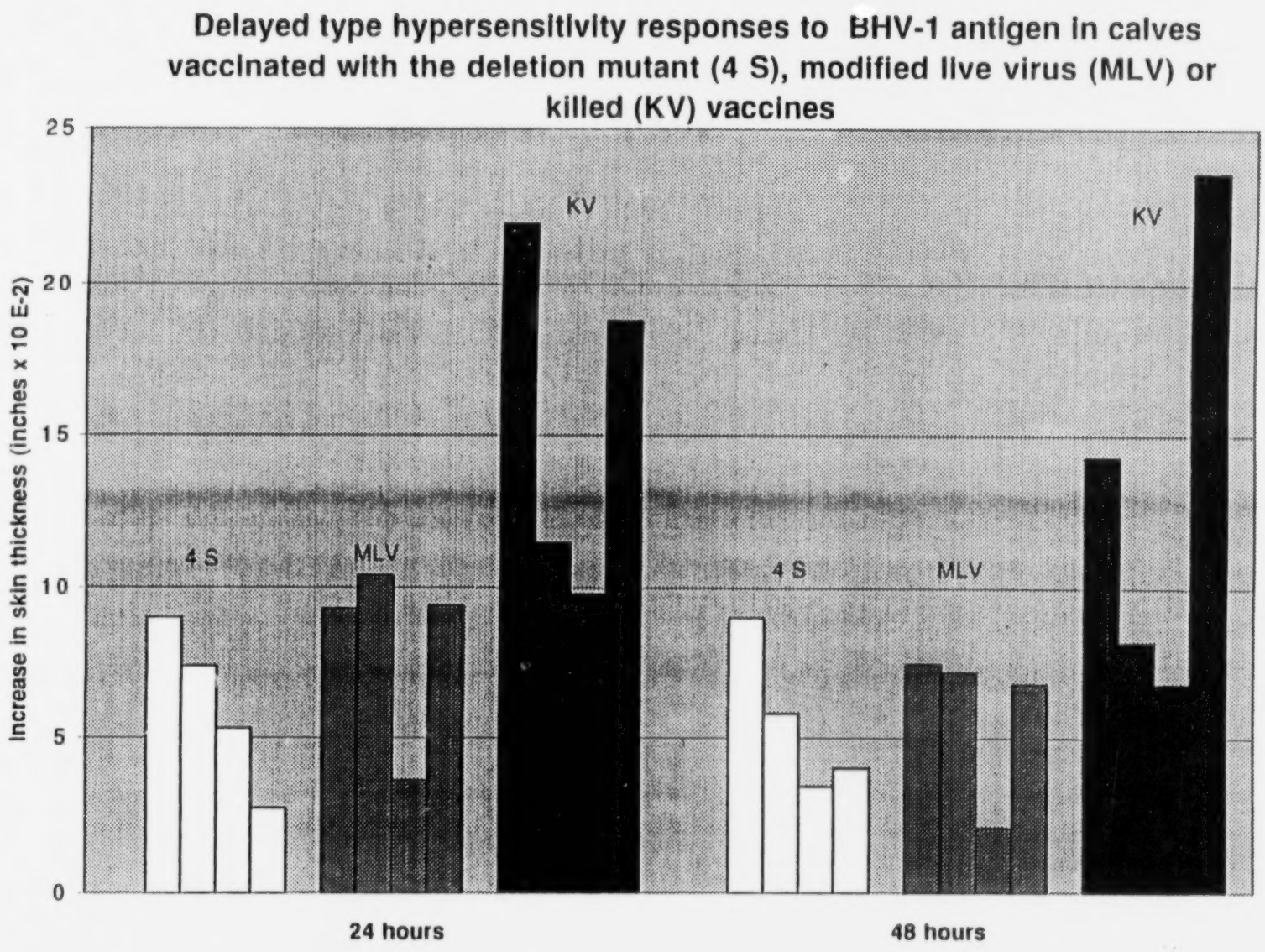
Interferon gamma responses to BHV-1 antigen in calves vaccinated with the deletion mutant (4 S), modified live virus (MLV) or killed (KV) vaccines on day 7 following secondary immunization




18 hour BHV-1 cytotoxicity assay (E:T=100:1), day 7 following secondary immunization in calves vaccinated with the deletion mutant (4 S), modified live virus (MLV) or killed (KV) vaccines
% Cytotoxicity
35
30
25
20
15
10
5
0
4 S
MLV
KV

Delayed type hypersensitivity responses to BHV-1 antigen in calves vaccinated with the deletion mutant (4 S), modified live virus (MLV) or killed (KV) vaccines




Delayed type hypersensitivity responses to BHV-1 antigen in calves vaccinated with the deletion mutant (4 S), modified live virus (MLV) or killed (KV) vaccines
Increase in skin thickness (inches x 10 E-2)
25
20
15
10
5
0
4 S
MLV
KV
4 S
MLV
KV
24 hours
48 hours

Appendix 2: Results: Experiment #2

BHV-1 antibody levels measured by ELISA in nonvaccinated calves (Control) and calves vaccinated with BHV-1 killed virus (KV) or deletion mutant virus (4S) vaccines.

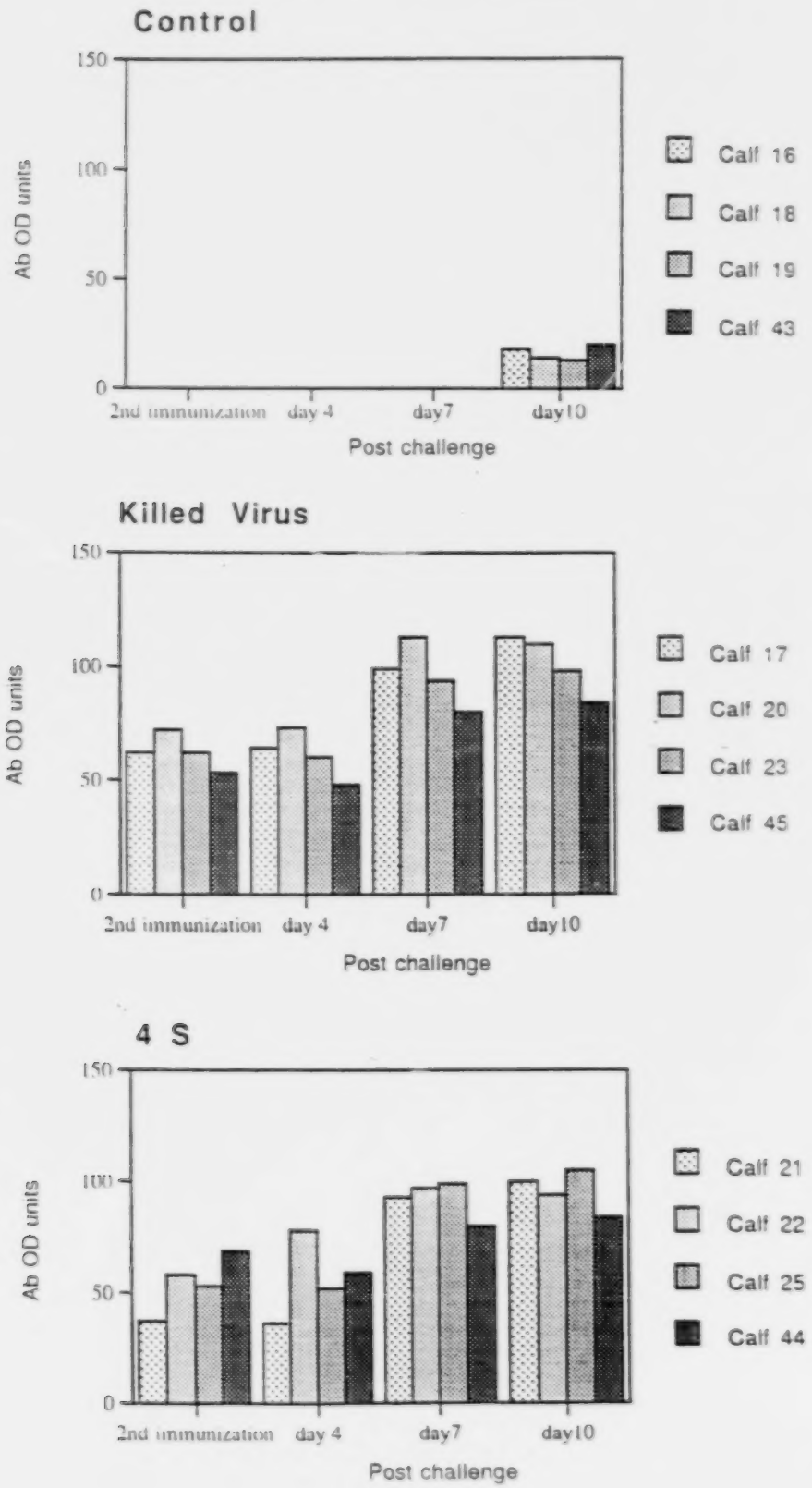
Blastogenesis responses to BHV-1 antigen in nonvaccinated calves (Control) and calves vaccinated with BHV-1 killed virus (KV) or deletion mutant virus (4S) vaccines on day 14 following secondary immunization.

Interferon gamma responses to BHV-1 antigen in nonvaccinated calves (Control) and calves vaccinated with BHV-1 killed virus (KV) or deletion mutant virus (4S) vaccines on day 10 following challenge.

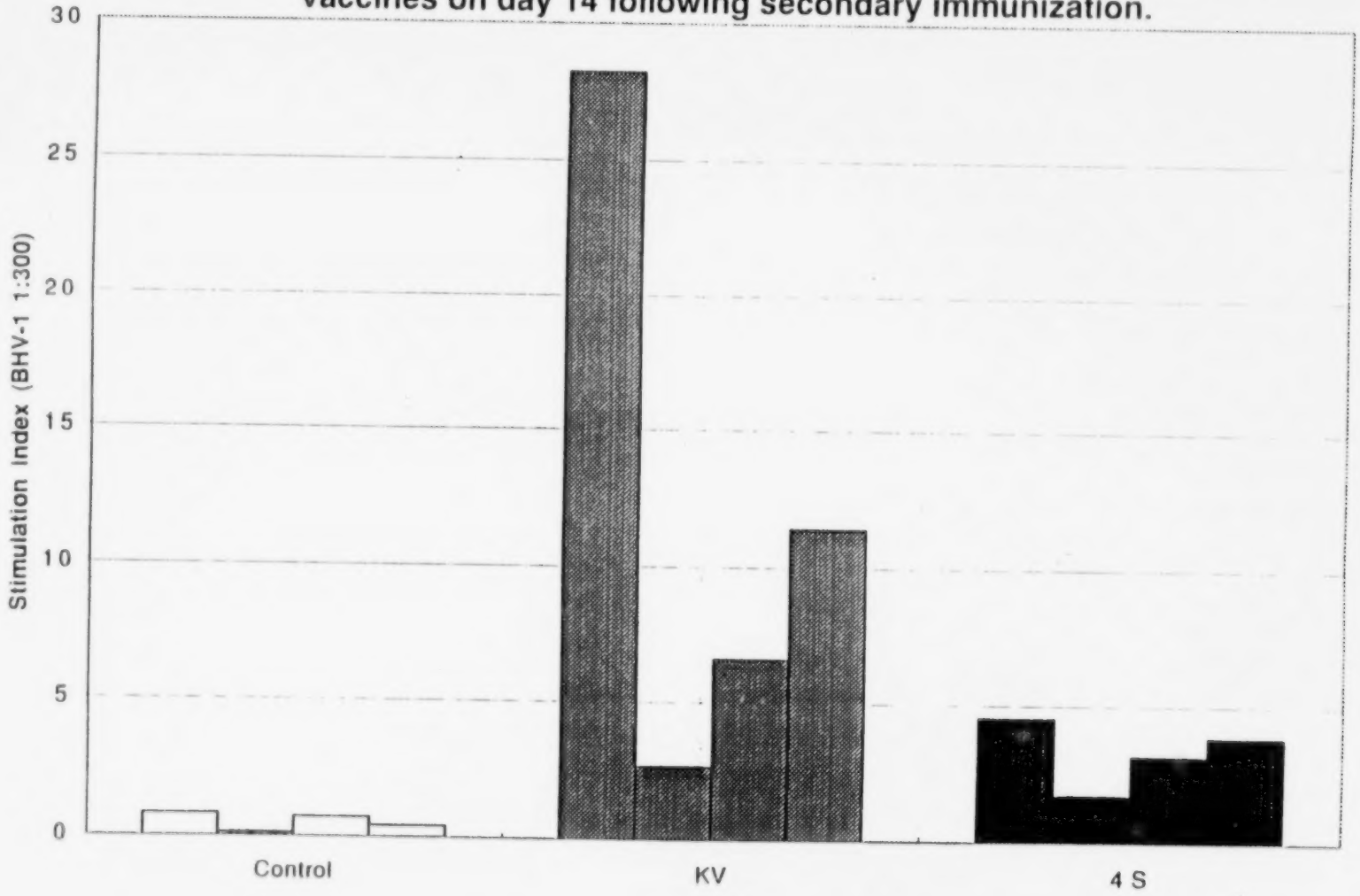
18 hour BHV-1 cytotoxicity (E:T 100:1) responses in nonvaccinated calves (Control) and calves vaccinated with BHV-1 killed virus (KV) or deletion mutant virus (4S) vaccines on day 14 following secondary immunization.

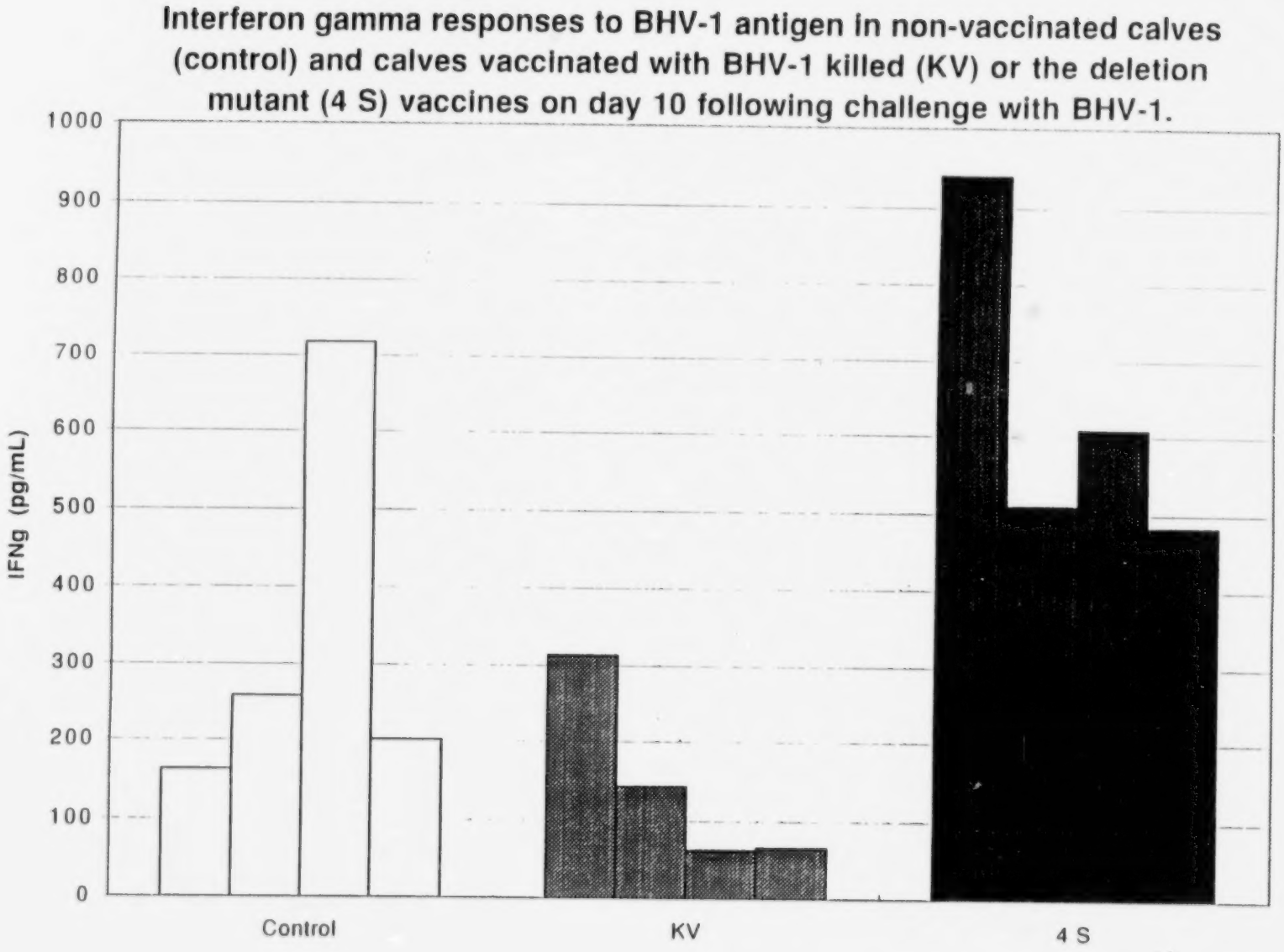
Delayed type hypersensitivity responses to BHV-1 antigen in nonvaccinated calves (Control) and calves vaccinated with BHV-1 killed virus (KV) or deletion mutant virus (4S) vaccines.

BHV-1 antibody levels measured by ELISA in non-vaccinated calves (Control) and calves vaccinated with BHV-1 killed (KV) or deletion mutant (4 S) vaccines

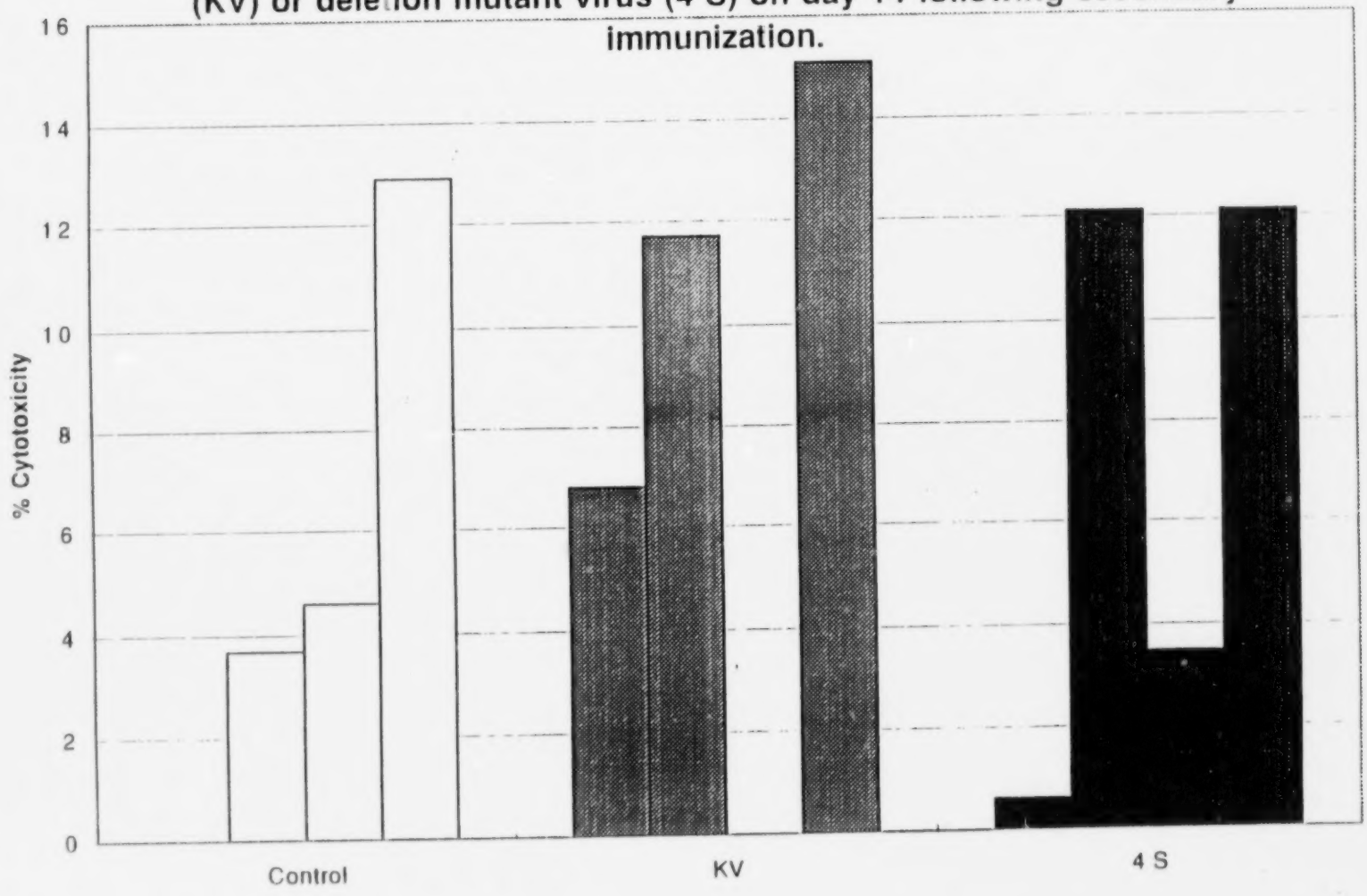


Blastogenesis responses to BHV-I antigen in non-vaccinated calves (Control) and calves vaccinated with BHV-1 killed (KV) or deletion mutant (4 S) vaccines on day 14 following secondary immunization.

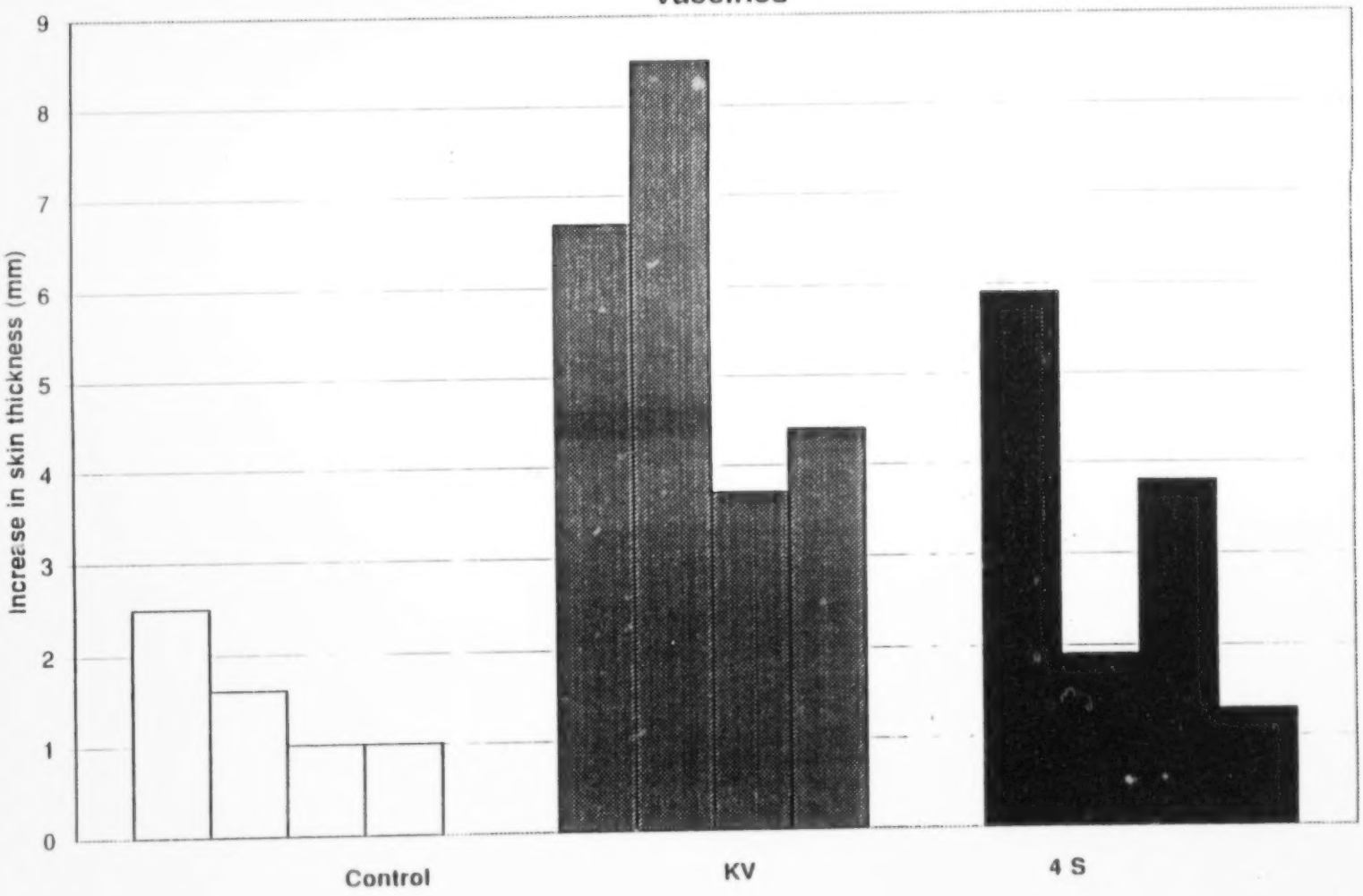



Interferon gamma responses to BHV-1 antigen in non-vaccinated calves (control) and calves vaccinated with BHV-1 killed (KV) or the deletion mutant (4 S) vaccines on day 10 following challenge with BHV-1.
IFNg (pg/mL)
1000
900
800
700
600
500
400
300
200
100
0
Control
KV
4 S

18 hour cytotoxicity (E:T=1:100) responses to BHV-I antigen in non-vaccinated calves (Control) and calves vaccinated with BHV-1 killed virus (KV) or deletion mutant virus (4 S) on day 14 following secondary immunization.



Delayed type hypersensitivity responses to BHV-1 antigen in non-vaccinated calves and calves vaccinated with BHV-1 killed (KV) or deletion mutant (4S) vaccines

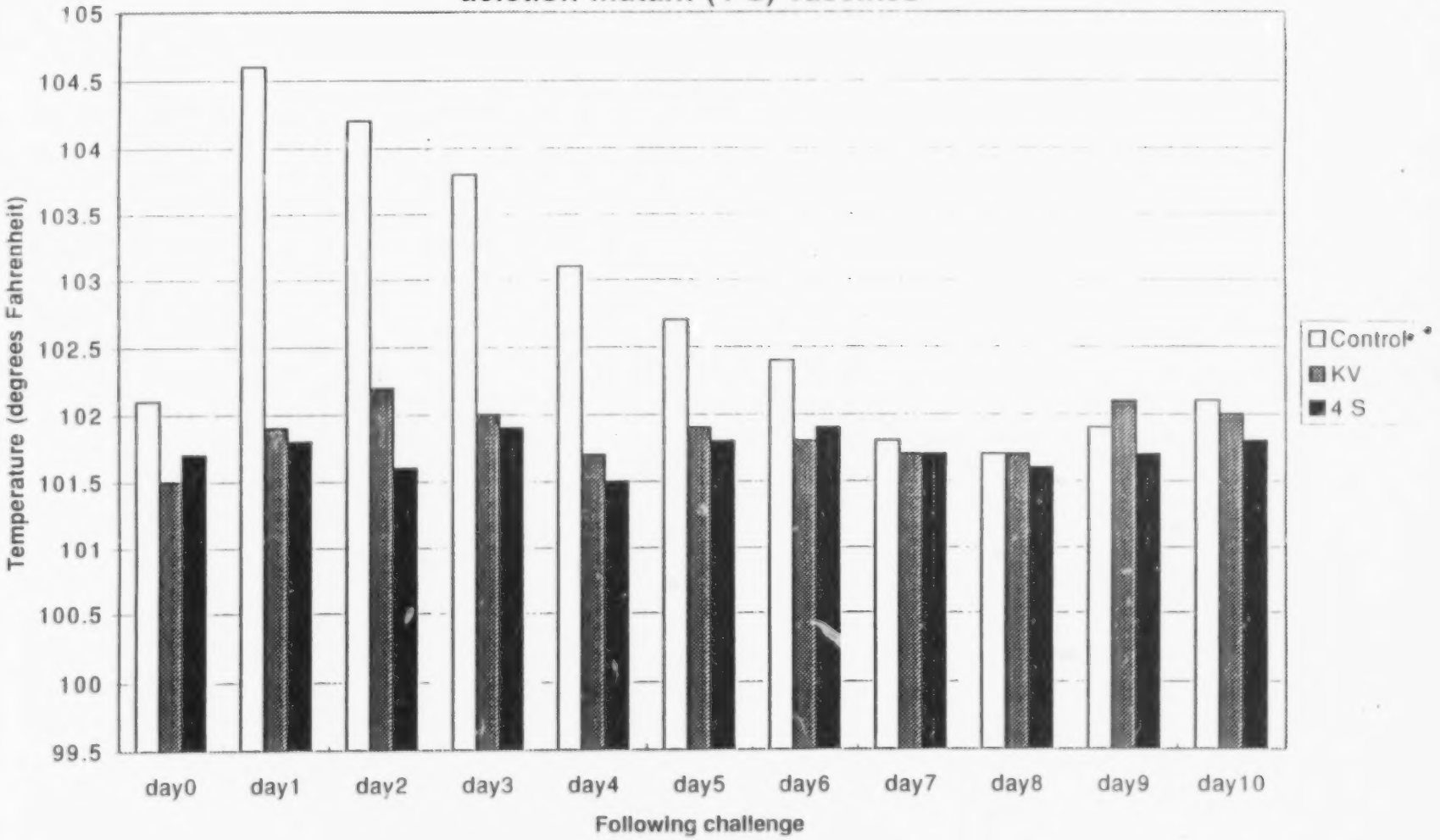


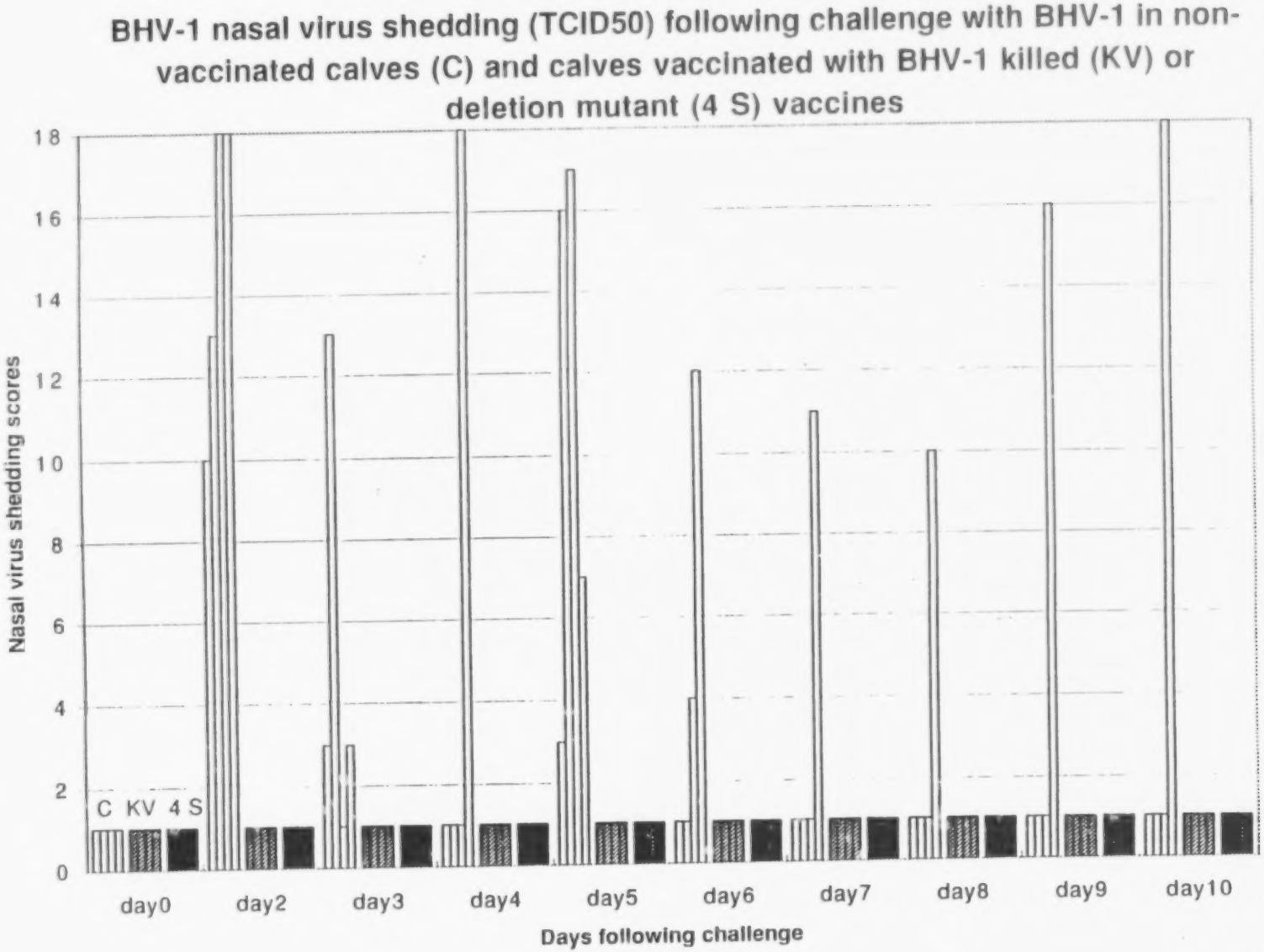
Mean daily body temperatures following challenge with BHV-1 in nonvaccinated calves (Control) and calves vaccinated with BHV-1 killed virus (KV) or deletion mutant virus (4S).

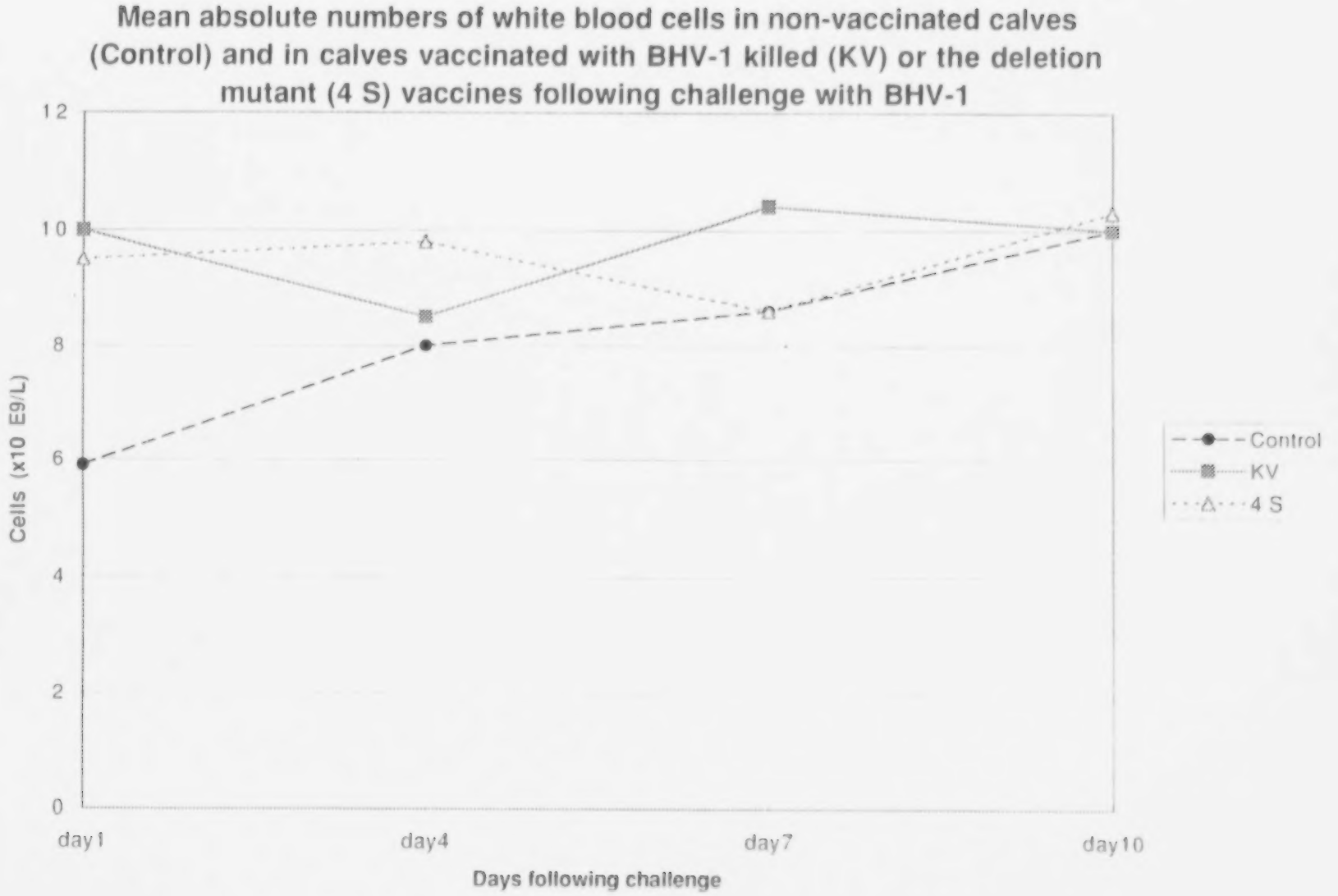
BHV-1 nasal shedding ($TCID_{50}$) following challenge with BHV-1 in nonvaccinated calves (Control) and calves vaccinated with BHV-1 killed virus (KV) or deletion mutant virus (4S).

Mean absolute numbers of white blood cells in nonvaccinated calves (Control) and calves vaccinated with BHV-1 killed virus (KV) or deletion mutant virus (4S) vaccines.

Mean daily body temperatures following challenge with BHV-1 in non-vaccinated calves (control) and calves vaccinated with killed (KV) or deletion mutant (4 S) vaccines




BHV-1 nasal virus shedding (TCID50) following challenge with BHV-1 in non-vaccinated calves (C) and calves vaccinated with BHV-1 killed (KV) or deletion mutant (4 S) vaccines
Nasal virus shedding scores
0
2
4
6
8
10
12
14
16
18
C KV 4 S
day0
day2
day3
day4
day5
day6
day7
day8
day9
day10
Days following challenge


Mean absolute numbers of white blood cells in non-vaccinated calves (Control) and in calves vaccinated with BHV-1 killed (KV) or the deletion mutant (4 S) vaccines following challenge with BHV-1
12
10
8
6
4
2
0
Cells (x10 E9/L)
day1
day4
day7
day10
Days following challenge
Control
KV
4 S